



Enhancement of Lymphatic Vessels in the Superficial Layer in a Rat Model of a Lymphedematous Response

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Background: The morphologic and histologic behavior of lymphatic vessels in lymphedema has not been well analyzed using laboratory animals. The purpose of the present study was to elucidate the regeneration process of lymphatic vessels after acute lymphedema in a rat model.

Methods: The acute lymphedema was induced by an amputation and a replantation surgery on a rat hind limb. Recovery of lymphatic flow was traced using fluorescent lymphography with dye injection. The morphology and number of lymphatic vessels were immunohistochemically detected and quantified in both superficial and deep layers.

Results: The swelling was the most severe, and the number of lymphatic vessels in the superficial layer was significantly and maximally increased on postoperative day 3. Backflows and overflows were also detectable in the superficial layer on postoperative day 3. The number of lymphatic vessels had decreased but remained significantly higher than that in the controls on postoperative day 14, when the swelling decreased to the levels in the controls. In contrast, the number of lymphatic vessels in the deep layer showed a tendency toward increased numbers; however, it was not statistically significant on postoperative day 3, 7, or 14.

Conclusions: We have obtained solid evidence showing the differential potency of lymphatic vessels between the superficial and the deep layers after temporal lymphedematous induction. Further analysis of lymphedematous responses in animal models could provide new insights into the challenges associated with the clinical treatment of lymphedema. (*Plast Reconstr Surg Glob Open* 2018;6:e1770; doi: 10.1097/GOX.0000000000001770; Published online 25 May 2018.)

INTRODUCTION

The lymphatic system plays an important role in maintaining the homeostasis of tissue fluid, immune cell trafficking, and absorption of dietary lipids. Lymphatics are

present in the skin and almost all internal organs excluding the central nervous system, bone marrow, and avascular tissues such as the epidermis. The lymphatic network drains interstitial fluid from the tissues and returns it to the vascular system. Aberrant lymphangiogenesis is associated with the pathogenesis of human disorders including lymphedema, tumor metastasis, and inflammatory conditions such as asthma, psoriasis, and rheumatoid arthritis.^{1,2}

We must collect analytical findings and information concerning lymphatic flow at both the experimental and clinical levels when examining clinical treatments for lymphedema. Lymphoscintigraphy is a gold standard for diagnosis when pathological changes of lymphatic vessels must be identified. Computed tomography and fluorescence lymphographies can provide views of lymph flow in detail.^{3–6} However, only partial lymphatic pathways were detectable by such methods after the uptake of contrast agents, but whole vessels were not visible. There is little information about the horizontal anatomy concerning the localization of lymphatic vessels in the superficial and/or deep layers. The number of lymphatic vessels localizing

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in horizontal levels and in which layers and regions the lymphatic vessels distribute is inconsistent. To our knowledge, there have been no reports about lymphatic distribution in deep regions. The distribution of all lymphatic vessels in horizontal cross-section must be elucidated first in laboratory animals.^{7,8} Intradermal or subdermal lymphangiogenesis has been studied morphologically, whereas physiological and pathological lymphatic responses in the deep layers, such as the intramuscular and peri-muscular layers including deep fascia, are still unknown. An adequate animal model to study lymphedema could be very powerful to help reveal the molecular and cellular backgrounds underlying lymphangiogenesis and in developing further treatments for human clinical lymphedema from a novel viewpoint.⁹⁻¹²

In the present study, we tried to elucidate the lymphatic distribution in the superficial and deep layers of the lower leg, and then tried to trace the course of lymphangiogenesis in an acute lymphedema animal model after an amputation and replantation procedure. Our animal model cannot repeat human clinical lymphedema completely; however, important information needed to solve clinical problems could be realized at this experimental level.

MATERIALS AND METHODS

Acute Lymphedema Model

Adult male Wistar rats (SLC, Shizuoka, Japan) weighing 250–350 g were used in this study. All animal experiments were conducted in strict accordance with institutional and NIH guidelines for “Using Animals in Intramural Research,” and all experimental protocols were approved by the Animal Research Committee of Okayama University, Japan (No. OKU-2014176). All rats were intraperitoneally

injected with pentobarbital sodium (Dainippon Sumitomo Pharma. Co., Osaka, Japan) at 50 mg/kg of rat body weight for anesthesia, and their hair was carefully removed with depilatory around the surgical area of the legs.

We evaluated the lymphatic pathways following an acute lymphedema model of the rat hind limb. The hind limbs of the anesthetized rats were amputated around the right groin line. The hind limb was cut so that the groin lymph node was contained in the central side, and the popliteal lymph node was contained in the peripheral side (Fig. 1). Soon after the amputation, the replantation surgery was carried out using an allograft. First, the femoral bone was fixed with a 20-G needle. The femoral artery and vein were anastomosed using 10-0 nylon. The muscles and skin were sutured with 3-0 silk. The lymphatic vessels were not anastomosed. The operated rats were caged individually with ad libitum access to food until they underwent the following processes. They were put in Elizabethan collars so as not to bite themselves.

Tissue Harvest and Immunohistochemical Staining of Lymphatic Vessels

The rats were anesthetized and perfused transcardially with 4% paraformaldehyde fixative. The rats' hind limbs were amputated around the right groin line. The amputated lower extremities were fixed again in 10% neutral buffered formalin overnight, defatted in ethanol for 4 days, and then decalcified by soaking in 10% ethylenediaminetetraacetic acid (EDTA) for 1 month. The tissues were cut in at 5 mm peripherally from the groin line (or from the suture line). All the histochemical analyses were carried out on the sections at the peripheral part in reference to the suture line.

The tissues were embedded in paraffin, and 4.5-μm thick sections were prepared as whole horizontal sections

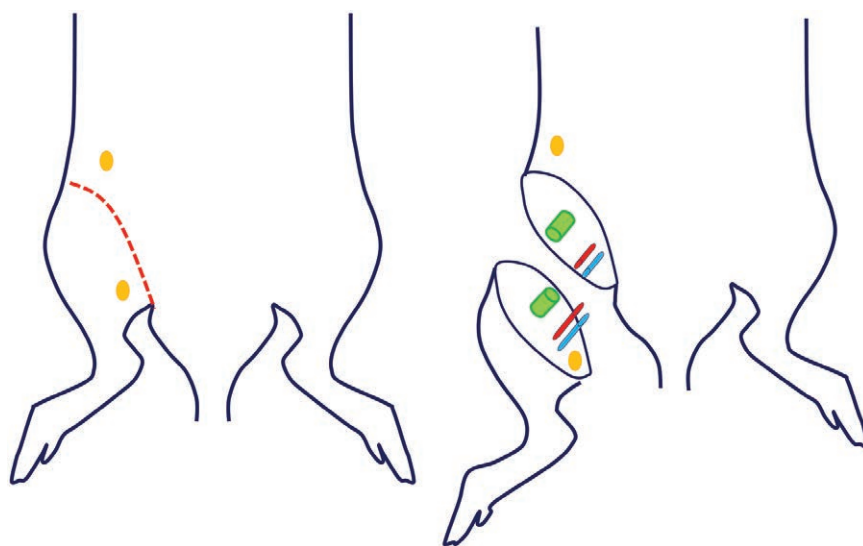


Fig. 1. Hind limb amputation and replantation procedures. Amputation was carried out between the inguinal lymph node (upper yellow mark) and the popliteal lymph node (lower yellow mark). The femoral bone (green), the femoral artery (red), and the femoral vein (blue) were cut once. The femoral bone was fixed internally, the femoral artery and vein were anastomosed microscurgically, and then the muscle, subcutaneous tissue, and skin were sutured layer to layer.

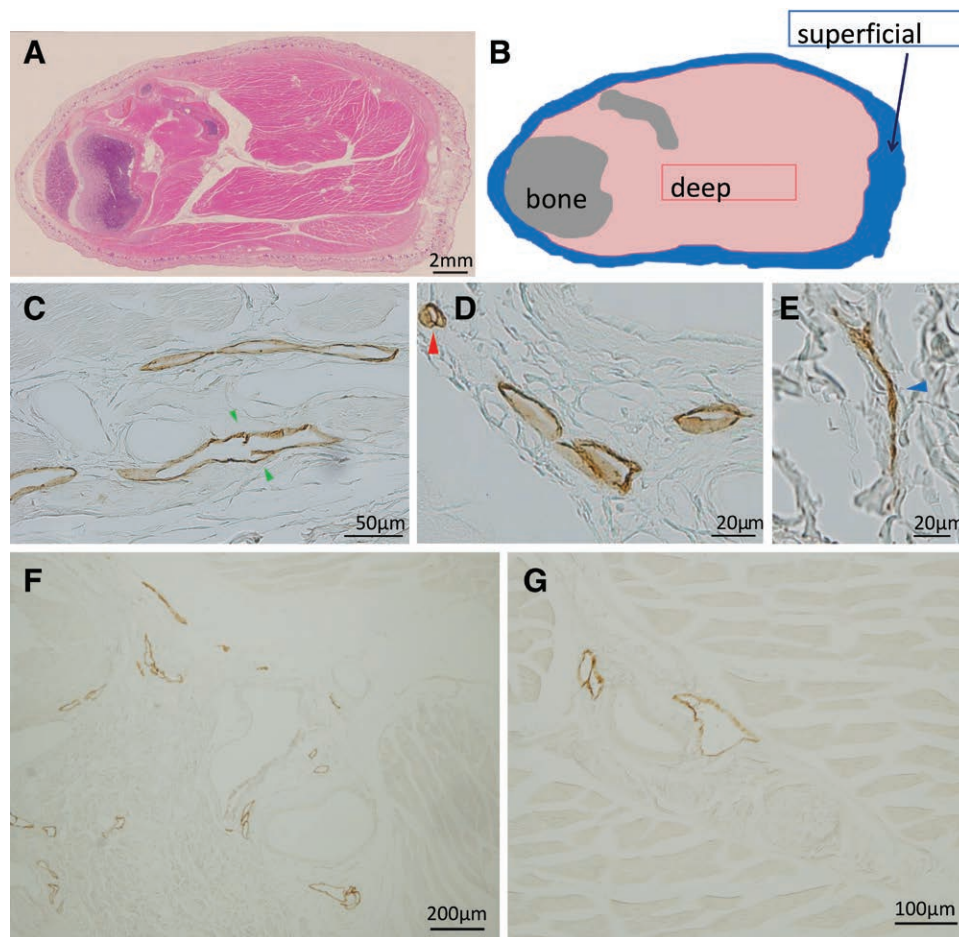


Fig. 2. Immunohistochemical studies of lymphatic vessels reacted with Podoplanin antibody on POD 0. A, Hematoxylin and eosin staining of a horizontal slice section of rat hind limb at a lower magnification. B, We defined “the superficial layer” as the dermis and the hypodermis layers including the epimysium, and “the deep layer” as the subfascial layer excluding the epimysium. Lymphatic vessels detected in the superficial layer are shown in the center, and the deep layer in the area below. C, D, and E, Podoplanin-positive lymphatic vessels in the superficial layer: The lymphatic valves were detectable (green arrowheads). There were lymphatic vessels smaller than 10 µm diameter (red arrow head) and squashed or linear-shaped immunopositivity (blue arrowhead). This staining pattern and the immunopositive debris were undetectable as lymphatic vessels in this study. In the deep layer, immunopositivities were also dense between the periosteum and muscles on the medial side of the femur (F). In the muscular layer, the lymphatic vessels were observed in the neurovascular bundles (G).

of the hind limb. The sections were then deparaffinized in xylene and rehydrated. After antigen retrieval and blocking, sections were incubated with anti-rat podoplanin monoclonal antibody (11035 AngioBio Co., San Diego, Calif.) overnight at 4°C.¹³ Next, sections were incubated in antimouse IgG Horse Radish Peroxidase conjugated (414171 Nichirei Co., Tokyo, Japan). The immunopositivities were visualized using a 3-3'-diaminobenzidine tetrahydrochloride Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, Calif.). Finally, the sections were dehydrated and mounted. In the normal and postoperative days (PODs) 3, 7, and 14 subject rats (each n = 3), we manually counted all the immunopositive lymphatic vessels in the horizontal cross sections. We defined “the superficial layer” as the dermis and the hypodermis layers including the epimysium, and “the deep layer” as the

subfascial layer excluding the epimysium. The number of lymphatic vessels was counted manually in 4 independent specimens from each animal.

Measuring of the Ankle Circumference

The ankle circumferences were measured manually before and after the replantation procedure on PODs 1, 3, 5, 7, 10, and 14 (n = 5). We defined POD 0 as the control before the operation. The mean circumference was obtained by taking the average of 10 measurements from 1 sample.

Fluorescence Lymphography with Indocyanine Green

The near-infrared fluorescence imager PDE (Hamamatsu Photonics Co., Hamamatsu, Japan) was used to observe lymphatic flow. We injected 0.02 ml of 5 mg/ml indocyanine green (ICG) (Diagnogreen, Daiichi-Sankyo,

Tokyo, Japan) intradermally into the rats' hind toes using a 30-gauge needle.¹⁴ Six rats were observed from POD 0 to POD 28. Fluorescence images were taken up to 120 minutes after the ICG injections.

Dye Injection Procedure

The lymphatic pathways were visualized after the dye injection procedure as described previously.¹⁵ In this study, we manually injected acrylic ink (Sakura Acryl Colors, Sakura Color Products Co., Osaka, Japan) that had been diluted in saline directly into the subdermal capillary lymphatic vessels of the dorsalis pedis. The dye was delivered from the capillary lymphatic vessels to the collecting lymphatic vessels in the superficial layer, but was not delivered to the lymphatic vessels in the deep layer of the lower leg. Next, the dye was observed to ascend immediately to the inguinal region and the intraperitoneal lymph nodes. The skin was carefully removed to observe the subdermal lymphatic vessels directly. We injected the dye directly into the lymphatics of the superficial tissues, and we performed histological examinations of them on PODs 0, 3, and 7.

Statistical Analysis

Statistical comparisons of the ankle circumferences and the number of lymphatic vessels were carried out using the Student's unpaired *t* test. Statistical significance was set at *P* value less than 0.05 and 0.01. All numerical data are presented as the mean \pm SD.

RESULTS

The Normal Distribution of Lymphatic Vessels in Horizontal Whole Sections of the Lower Leg

In normal conditions, we examined the distribution and morphology of the lymphatic vessels, considering the superficial layer and the deep layer separately. The mean number of preoperative lymphatic vessels on POD 0 was 70.6 ± 8.4 in the superficial layer and 190 ± 32.5 in the deep layer. Various shapes and sizes of immunopositive vessels were detected in linear, nearly circle, irregular circle, or elliptical forms, and their diameters ranged from less than 10 μm to more than 100 μm (Fig. 2). We defined such immunoreactivity with cavities as the lymphatic vessels based on their morphology, however could not define the linear type as a collapsed lymph duct in this study (Fig. 2E center, right). The linear staining pathways were not conspicuous in the deep layer, but were in the superficial layer. In the deep layer, immunopositivities were also dense between the periosteum and muscles on the medial side of the femur. In the muscular layer, the lymphatic vessels were observed in the neurovascular bundles or along the small vessels.

ICG lymphography and dye injection procedures showed evidence of lymphatic linear pathways from the dorsalis pedis to the dorsal side of the hind limb into the popliteal lymph node area (Fig. 3A) and then on into the peritoneal cavity. No ICG-fluorescence or dye leakage from lymphatic ducts was detected.

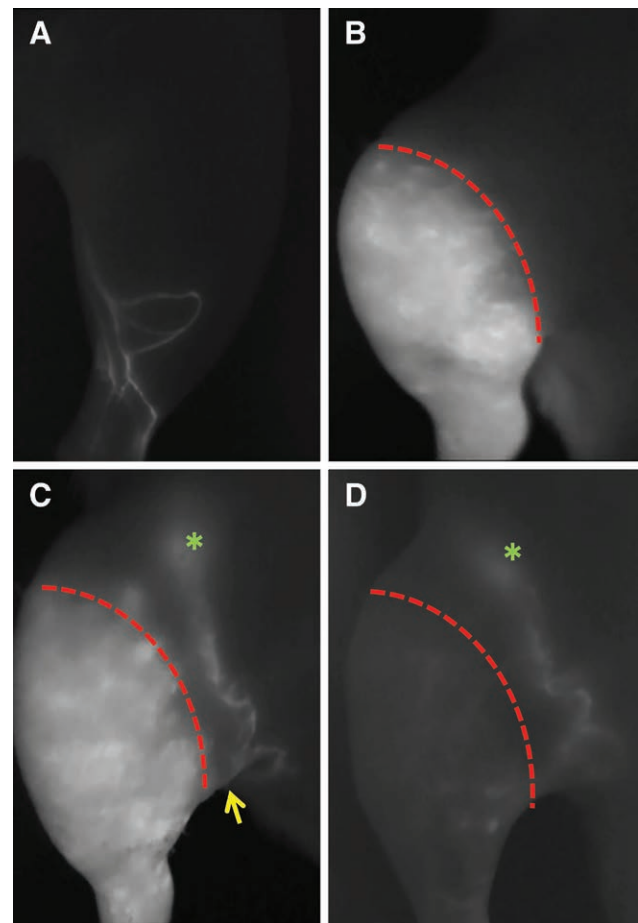


Fig. 3. Time-dependent change of ICG-lymphangiography before and after the surgery. A, Preoperative lymphangiography: lymph flows were detectable on the dorsal side. B, POD 2: ICG-fluorescence was stuffed at the distal part from the suture line (red line). No fluorescence was detected beyond the proximal part. C, POD 5: ICG-fluorescence was detectable across the suture line (detected by yellow arrow). Stuffed fluorescence was also detectable. The inguinal lymph node was fluoro-positive (indicated by green asterisks). D, POD 14: Stuffed fluorescence was washed out from the distal part of the hind limbs. Fluoro-positive lymph flows across the suture line to the inguinal lymph node (indicated by green asterisks) were detectable.

Temporal Change of Lymphatic Vessels in Acute Lymphedema

Edematous change achieved a peak on POD 3 (Fig. 4). At this point, in ICG lymphography, the area in the periphery compared with the suture line showed uniformly high fluoro-intensity representing dermal backflow on both the ventral and dorsal sides. ICG-fluorescence was not detected across the suture line (Fig. 3B, indicated by red line). The dye injection procedure revealed back-flows from the collecting vessels to the capillary vessels, and overflows to the inter-tissue spaces. The number of lymphatic vessels in the superficial layer increased significantly (Figs. 5, 6). On the other hand, in the deep layer, the number of immunopositive-staining areas showed a tendency to increase from the preoperative status. However, this increase was not statistically significant (Fig. 5A).

On and after POD 5, the swelling started to decrease but still enlarged significantly (Fig. 4). During this period,

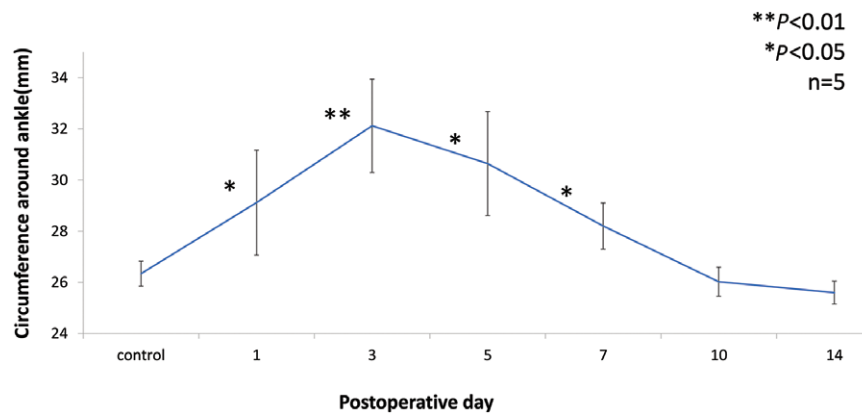


Fig. 4. The ankle circumference temporally increased after the surgery. The circumference reached a maximum on POD 3 and returned to baseline on POD 10. All data were compared with POD 0. ** $P < 0.01$, * $P < 0.05$. $n = 5$.

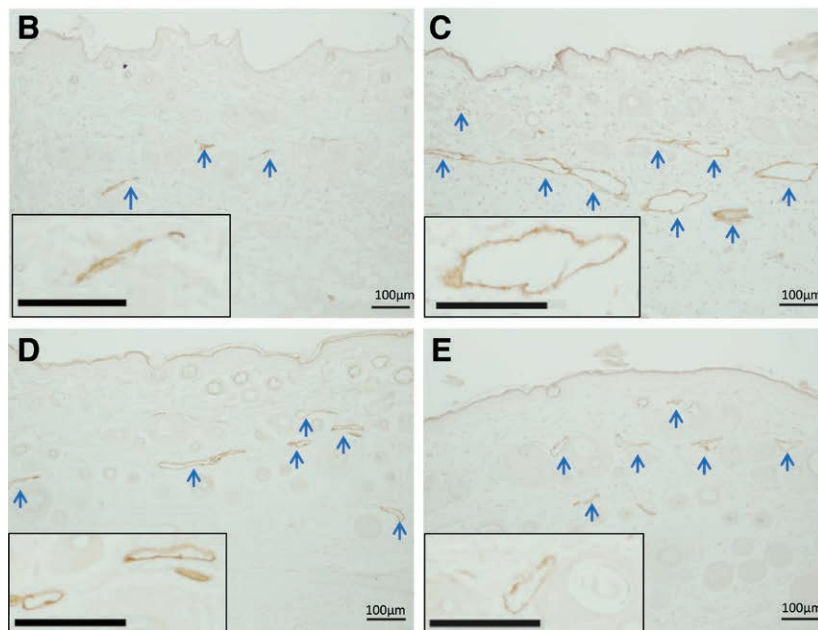
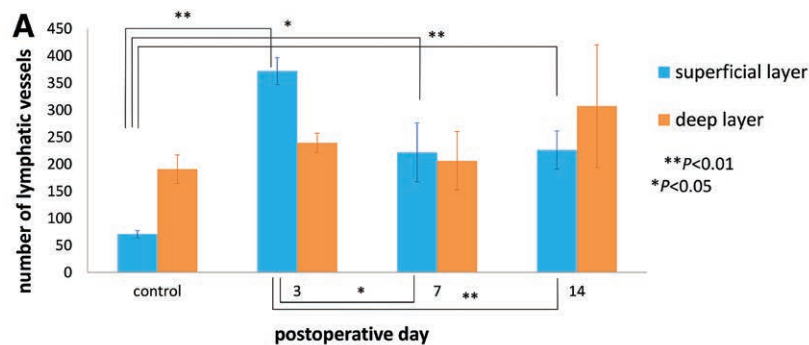


Fig. 5. Alteration in the distribution of the lymphatic vessels after the replantation. A, Comparison of the numbers of podoplanin-positive lymph vessels in the superficial and the deep layers. The number of lymph vessels in the superficial layer significantly increased on PODs 3, 7, and 14. ** $P < 0.01$, * $P < 0.05$. $n = 12$. B, Podoplanin-immunopositive lymphatic vessels in the superficial layer on POD 0. C, The number and the size of lymphatic vessels increased and expanded on POD 3. D, The number and the size of lymphatic vessels still increased and expanded on POD 7. E, Lymphatic vessels remained increased on POD 14. Higher magnification microphotographs have been inserted to show immunopositivities (inserts in each photograph, Bar = 100 μm).

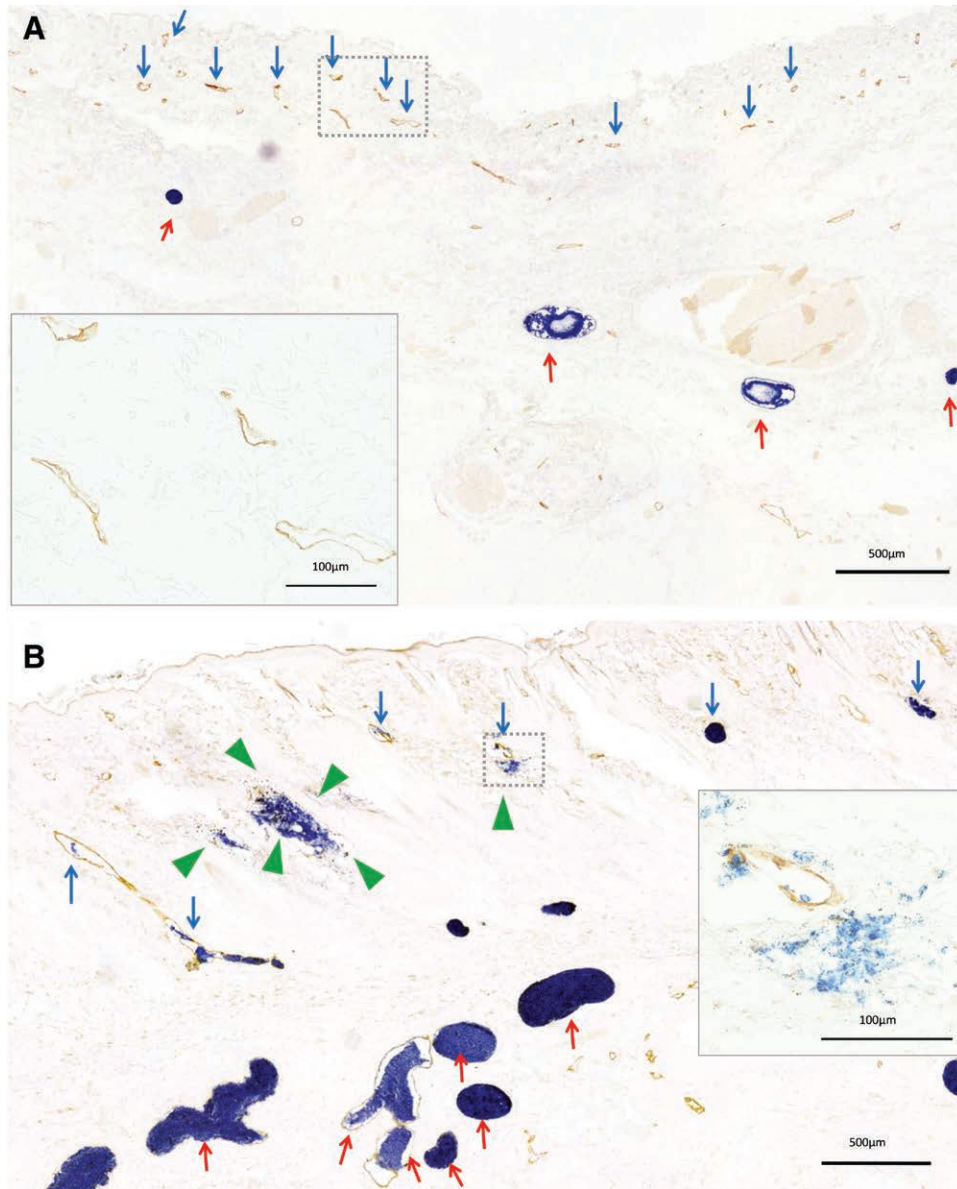


Fig. 6. Swelling lymphatic vessels in the superficial layer after replantation surgery. A, POD 0: dye filled in the collecting lymphatic vessels (indicated by red arrows). There was no leakage to the extraductal regions and no backflow to the dermal or subdermal capillary lymph vessels (indicated by blue arrows). B, POD 3: Collecting lymphatic vessels were overfilled with dye and were swelling (red arrows). Capillary vessels filled with the ink (indicated by blue arrows) due to back-flows were detectable. Green arrows show overflow to the extravascular space of lymphatic capillary. Higher magnification microphotographs have been inserted to show immunopositivities (inserts in each photograph).

ICG lymphography showed recanalized pathways, and the ICG-fluorescence was still very noticeable on distal part of the suture line (Fig. 3C). The number of lymphatic vessels on POD 7 decreased with statistical significance compared with POD 3, but still remained increased significantly compared with the preoperative state (Fig. 5A).

On and after POD 10, the edema resolved to baseline (Fig. 4). At this point, ICG lymphography showed recanalization, and pooling of the fluorescence disappeared completely (Fig. 3D). Dye injection procedures on POD 14 also showed the recanalized pathway clearly extending across

the suture line (Fig. 7). The number of lymphatic vessels on POD 14 decreased with statistical significance compared with POD 3, but remained significantly increased from the preoperative state similar to POD 7 (Fig. 5A).

DISCUSSION

An Increase in the Number of Lymphatic Vessels in the Superficial Layer

From our study, we developed the following 2 hypotheses to explain the increase in the number of lymphatic

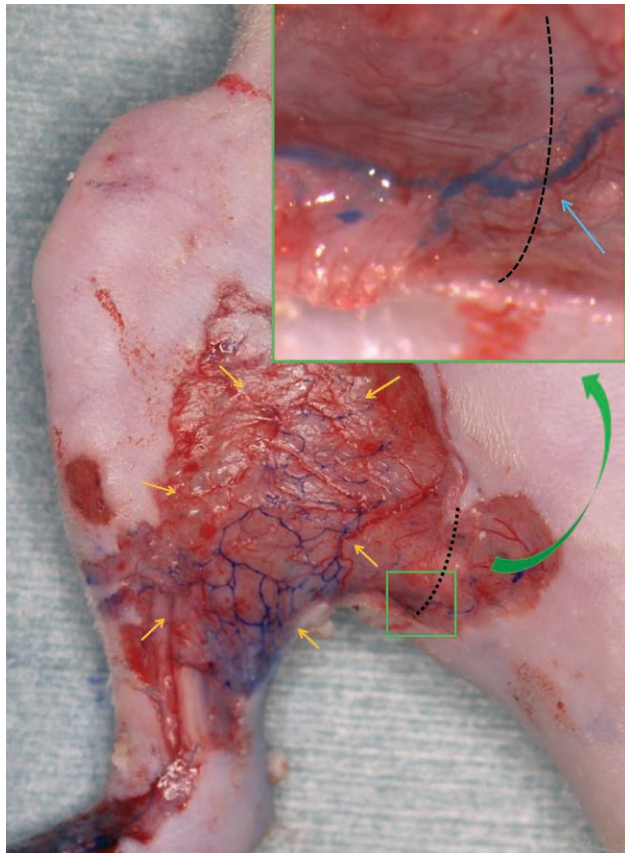


Fig. 7. Lymphatic vessels recanalized beyond the suture line. The lymphatic network was visualized with acrylic ink on POD 14. The dye ascended immediately to the inguinal region beyond the suture line. The lymphatic network on the peripheral side of the suture was enhanced (surrounded by yellow arrows). A recanalized superficial lymphatic vessel was observed clearly at a higher magnification (indicated by the blue arrow in the green square). The black dotted line shows the sutured line.

vessels. First, the reconstruction of lymphatic vessels was promptly stimulated after the operation. It is generally accepted that lymphangiogenesis is triggered by inflammation and retention of lymph fluid.^{9,16,17} Therefore, we have strongly considered the involvement of lymphangiogenesis in the retention of lymph flow in our system. However, as shown in Figure 3, we can also suggest the lymphatic drainage inosculature between donor and recipient lymphatic vessels near the suture site because of the rapid restoration of ICG flow in the recipients' main lymphatic trunk. Therefore, as our second hypothesis, we suggest that all the lymph vessels showed little or no change from before to after the operation; however, we were unable to detect the majority of the superficial lymphatic vessels on POD 0 by our methods. From our observations, debris, and/or linear staining with podoplanin-immunoreactivities were conspicuous in the superficial layer on POD 0, but we did not define them as lymphatic vessels (Fig. 2). The afferent lymphatic flow in the lymphatic capillaries might be too weak and too small to dilate the vessels. The lymphatic vessels might collapse to linear configurations or smaller sizes under normal conditions. After surgery,

such vessels might swell and then become detectable by our methods.

On POD 3, the reason for the increasing number of lymphatic vessels could be explained by the second hypothesis mentioned above. We have shown that the temporal retention of lymphatic fluid occurred along the swelling around the ankle after the amputation and replantation surgery, evidenced by the use of ICG lymphangiography and the dye injection method. This lymphatic retention also brought back-flow to the capillary lymph vessels and overflow to the interstitial spaces by extrusion of lymphatic fluids from the lymphatic collecting vessels on POD 3 (Fig. 6B below). Therefore, previously collapsed lymph vessels became dilated, and we could then detect them by podoplanin-immunostaining. We observed that the number of lymphatic vessels increased on POD 3.

We detected parallel relationships between the retention of lymphatic fluids detected by ICG lymphangiography (Fig. 3) and the ankle circumference (Fig. 4). We also detected parallel relationships between the ankle circumference and the number of lymphatic vessels in the superficial layer until POD 3. On POD 7, the swelling was decreasing (Fig. 4), and ICG retention below the suture line was also decreasing (Fig. 3); however, the number of lymphatic vessels in the superficial layer remained increased. These discrepant findings fit the first hypothesis mentioned above. Newly constructed lymphatic networks in the superficial layer after lymphangiogenesis was already occurring. On the other hand, the once dilated lymph vessels started to collapse again on POD 7.

Our data on ICG-lymphangiography (Fig. 3) clearly show that the lymphatic fluids were already flowing from the periphery beyond the suture line back to the midline after POD 7. Therefore, we could recognize that swelling did not depend on the actual number of lymph vessels, but on the lymphatic flows from the periphery to the central body parts.

Taken together with these hypotheses, we can speculate that the previously collapsed lymphatic vessels dilated around POD 3, and then collapsed again after PODs 7–14. The newly constructed lymphatic vessels after lymphangiogenesis might have contributed to the increased number of lymphatic vessels on PODs 7 and 14.

In the Deep Layer, There Was No Significant Increase in the Number of Lymphatic Vessels

From our results, the lymphatic vessels in the deep layer did not significantly increase in acute lymphedema. We were unable to detect the collapsed shape and linear staining using the podoplanin antibody in the deep layer at any time point. Histologically, the superficial layer (the dermis) has a rich network of small and thin lymphatic vessels. These branches descend to the deeper layer and join a larger and thick lymphatic trunk with relatively thick walls. In the normal state, the number of lymphatic vessels in the deep layer (190 ± 32.5) was approximately 3 times higher than in the superficial layer (70.6 ± 8.4) on POD 0 (Fig. 5). Stanton et al.¹⁸ clearly demonstrated by scintigraphy that lymph flow in the deep layer was ~2–3 times higher than that in the superficial layer in human

physiological conditions. Stanton's finding is consistent with our present data.

From these findings, we suggest that the potential for pathological retention of lymphatic fluid in the deep layer was weaker than in the superficial layer, and that after the amputation and replantation surgery, collapsed and linear lymph vessels would dilate only among the rich and thin networks of lymphatic drainage in the superficial layer.

We cannot completely rule out decreased blood flow as the main reason that the lymphatic flows brought on swelling despite the anastomoses of arteries and veins. However, we have obtained solid evidence of back-flows of lymphatic fluids and increased number of lymphatic vessels in the amputated and replanted legs.

In a recent clinic, we have tried to overcome lymphedema by lymphovenous anastomoses in the superficial layers mainly; however, the results were unfortunately inconsistent.³ To our knowledge, there are no challenges against lymph vessels focused on the deeper layer. Based on our present findings, the different behaviors of the lymph vessels in the superficial and deeper layers after a transient lymphedematous response present a hint as to how to treat lymphedema clinically and microsurgically in both the superficial and the deeper layers.

CONCLUSIONS

We have demonstrated a temporal lymphedematous response in the rat model. The rat's edema worsened on POD 3, but recovered to normal on POD 10. The number of lymph vessels increased during this acute phase but only in the superficial layer, not in the deeper layer. This increase still remained at a higher level after attenuation of the edema. We have discovered new findings showing the differences in activity between the lymphatic vessels in the superficial and the deep layers. Future attempts to enhance the recovery of lymph flows in our rat model after lymphovenous anastomosis may offer a potential strategy to cure lymphedema clinically, according to basic evidence on the anatomy and reconstitution of peripheral lymphatic vessels in the superficial and the deep layers.

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